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<b>(21) International Application Number:</b> PCT/US00/01044 <b>(22) International Filing Date:</b> 14 January 2000 (14.01.00) <b>(30) Priority Data:</b> 60/116,168      15 January 1999 (15.01.99)      US <b>(71) Applicant (for all designated States except US):</b> BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> RENNERT, Paul [US/US]; 278 Ridge Street, Millis, MA 02054 (US). <b>(74) Agent:</b> COX, Niki, D.; 14 Cambridge Center, Cambridge, MA 02142 (US).			<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ANTAGONISTS OF TWEAK AND OF TWEAK RECEPTOR AND THEIR USE TO TREAT IMMUNOLOGICAL DISORDERS			
<b>(57) Abstract</b>  The present invention relates to reagents which modify the activity of TWEAK and their use as therapeutic agents for the treatment of immunological disorders.			

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**ANTAGONISTS OF TWEAK AND OF TWEAK RECEPTOR AND THEIR USE TO TREAT IMMUNOLOGICAL DISORDERS**

5

**FIELD OF THE INVENTION**

The present invention relates to compositions and methods comprising reagents which bind to the novel protein TWEAK, and the use of TWEAK binding reagents to block the development of immunologic disorders. TWEAK binding reagents include monoclonal antibodies, as used herein to block the development of chronic Graft-  
10 Versus-Host Disease, soluble TWEAK-receptor-Ig fusion proteins, or other molecules which modify the binding of TWEAK to its' receptor(s). Other embodiments of the invention include reagents which bind to TWEAK receptor(s) to modify their activity, or modify the intracellular signaling of TWEAK receptor(s).

15

**BACKGROUND OF THE INVENTION**

Immunological disorders are manifested as a wide variety of diseases and pathologies, including autoimmune diseases, acute and chronic inflammatory disorders, organ transplant rejection, Graft-Versus-Host Disease (GVHD), lymphoid  
20 cell malignancies, septic and other forms of shock, loss of immune responsiveness as seen in HIV and SCIDS, and failure of the immune response to tumor growth.

Many immunological disorders are triggered by aberrant or uncontrolled responses to antigen. Autoimmune diseases are the result of the inappropriate response of the immune system to self-antigens, resulting in damage to cells and tissues. GVHD  
25 develops when donor cells from a bone marrow transplant (BMT) respond to host (ie. patient) antigens. Organ transplant rejection results when the patient's immune system responds to antigens derived from the transplanted organ. Acute inflammatory disorders such as hyper-allergic conditions and shock are the result of uncontrolled immune response to the triggering antigens.

30 T cell dependent immune responses require T cell recognition of antigen. For example, GVHD results from a complex interplay between donor T cells and host

immune system cells. The initiating event is the recognition by donor T cells of non-self, ie. host, antigens. These are referred to as alloantigens. Alloantigen recognition by these donor cells results in the production of immunoregulatory and inflammatory cytokines and chemokines, which advance and exacerbate the donor anti-host immune response. This disease can develop in either an acute or chronic form, depending on the regulation of complex cytokine networks which control the type of immune response which develops. Immune responses can be characterized as Th0, Th1, or Th2 depending on 1) the nature of the cytokines and chemokines produced by activated T cells during the response, and 2) the nature of the cytokines and chemokines produced by accessory and other cells during the response. Examples of non-T cells important during immune responses are B cells, dendritic cells, monocytes and macrophages, follicular dendritic cells, and endothelial cells. Together the cytokines produced influence a variety of cell types to differentiate, and the chemokines produced influence cell trafficking and localization. Th0 responses are characteristic of the short term stimulation of previously unstimulated (naive) T cells. Th0 T cells produce moderate amounts of a number of cytokines, notably Il-2 and TNF. Repetitively or chronically stimulated Th0 cells can differentiate into either Th1 or Th2 T cells, depending on a number of factors. Such factors include, but are not limited to, accessory cell cytokine production, the strength of T cell receptor engagement, and the nature of secondary signals received, eg. via the CD28 costimulatory receptor. In particular exposure of activated T cells to the cytokine Il-12, produced primarily by activated macrophages, supports differentiation to Th1 T cells, while exposure to Il-4 and Il-10 supports differentiation to Th2 T cells.

Th1 T cells produce cytokines such as Il-2 and IFN- $\gamma$  which are associated with inflammatory responses, T cell cytotoxicity, and macrophage activation. Th1 T cells respond to chemokines which attract cells into sites of tissue inflammation, such as Mip-1alpha, MIP-1beta, RANTES, IP-10, and MIG. Since cytotoxic T cells and activated macrophages act to eliminate damaged or infected cells, the Th1 response is responsible for controlling the immune response to intracellular pathogens. Importantly, production of Th1 chemoattractant chemokines such as IP-10 and MIG by macrophages and endothelial cells is closely regulated by IFN- $\gamma$ , which is the

prototypic chemokine produced by Th1 T cells. Thus feedback loops may develop between activated T cells and their environment, which augment the development of a particular type of response at a particular time and location

Th2 T cells produce cytokines such as Il-4, Il-5, Il-6, and Il-10 which support the development of humoral immune responses, including those which require the production of IgE, IgA, and IgG. These Ig responses are driven by the T cell mediated activation of B cells which "switch" their Ig phenotype from surface bound IgM and IgD to secreted Ig. Secreted Igs normally function to control infection from pathogens in circulation (IgG), at mucosal surfaces, such as the gut and oral cavity (IgA) and in the respiratory tract (IgE). Overproduction of Ig can cause disease, for example in SLE (IgG and IgA), allergic (Type I) hypersensitivity (IgE), and GVHD (IgG, IgA, and IgE). Th2 T cells also support the activation of eosinophils and Mast cells which can mediate acute responses to pathogens, for example in the respiratory tract. Th2 T cells respond to chemokines such as eotaxin and MDC, whose production is closely regulated by Il-4, the prototypic cytokine produced by NK1.1 and activated T cells.

The interaction of T cells with B cells is a complex and closely regulated process. To begin the activation process, B cells must receive an antigen signal through the B cell antigen receptor (membrane Ig). Secondly, B cells must receive specific contact dependent and contact independent signals from activated T cells. One required contact dependent signal is delivered via the binding of CD40L on T cells to CD40 on B cells. One required contact-independent signal is delivered by Il-4 secreted by activated T cells and by NK1.1 cells binding to the Il-4 receptor on B cells. These signals appear to take place within the T cell areas of secondary lymphoid organs, such as the spleen. The spleen, lymph nodes, tonsils, Peyer's patches and other secondary and tertiary lymphoid organs have distinct microanatomical areas within which T and B cells typically reside. All lymphocytes migrate out of the blood or the lymph into the T cell area of these organs first, by crossing endothelial cell layers such as the High Endothelial Venules in lymph nodes and Peyer's patches and the marginal sinus endothelial cell layer in the spleen. Then, the B cells move into B cell areas known as B cell follicles. B cells which have traversed the T cell area but have not become activated will leave the follicle after a few days. Activated B cell undergo a process of

differentiation. Some activated B cells, known as plasmacytes, secrete large amounts of antigen specific, low affinity IgM or IgG antibody. These B cells typically appear early after the induction of the immune response, and move into the red pulp of the spleen and other anatomical locations, where they persist, secreting antibody, for several days.

5 Other activated B cells differentiate within a region of the follicle known as the secondary follicle, or germinal center. Germinal centers form around networks of specialized antigen retaining cells known as Follicular Dendritic Cells (FDC), which are thought to display antigen to drive or refine the germinal center reaction. B cells within the germinal center have "switched" their Ig phenotype, and undergo "affinity  
10 maturation", with the result that they display a high affinity for their antigen target. Normally, the antigen target is a foreign antigen, although in diseases such as chronic GVHD and autoimmune disorders the Igs recognize self-antigens. Finally, B cells leave the follicles, migrate back through the T cells areas, and leave the organ via efferent circulation into the bloodstream.

15 B cells that have fully differentiated to express high affinity Ig are known as blast cells, and they leave B cell follicles to take up residence in various other environments, including the red-pulp areas of the spleen, the bone marrow, the liver, or mucosal cell layers lining the respiratory tract and gut. Some of these fully differentiated B cells are known as memory cells, and can persist for long periods of  
20 time, ready to respond to the same antigen if it is encountered again.

As B cells migrate from location to location within the lymphoid organs they require specific signals to guide them, and specific signals which ensure their survival. For example, multiple signals are required to maintain B cell follicular organization in the spleen. These include interaction of the BCA ligand with the chemokine receptor  
25 BLR-1, interaction of TNF with TNF-R55, and interaction of LTbeta with LTβ-R (reviewed in Chaplin and Fu, Current Opin. Immunol. 10: 298-297 (1998)). Mice which are deficient in any of these molecular pathways lose B cell follicular integrity in the spleen. Furthermore, all of these gene-deficient mice have also lost the ability to undergo germinal center reactions in the spleen. Disruption of other molecular  
30 pathways affects the germinal center reaction only. For example, mice deficient in CD40L maintain B cell follicles, but germinal centers do not form. B cells within the



germinal center environment require signals through CD40 to maintain survival and to downregulate IgM and switch to Ig expression.

B cells move not only within the follicle and germinal center, but also leave the follicle after activation, and move to other sites within the body. Memory B cells can be found in the bone marrow, and B cells which express IgA specifically traffic to cell layers in the gut and other mucosal sites. Other signals presumably guide activated T cells to specific sites within and between lymphoid compartments. For example, cytotoxic T cell can migrate to the site of infection or other antigen challenge to find and lyse their targets. The identities of all the signals which guide T and B cells between different microanatomic locations are not yet known. However it appears that multiple pathways orchestrate the T and B cell responses to antigen, both in secondary lymphoid organs, and at the sites of infection or inflammation.

GVHD is a well studied example of an antigen driven immune response. GVHD is an often fatal consequence of bone marrow transplantation (BMT) in human patients. The disease can occur in an acute or in a chronic form. Acute and chronic forms of GVHD are prototypic examples of the development of antigen specific Th1 and Th2 responses, respectively. The acute form of the disease occurs within the first 2 months following BMT, and is characterized by donor cytotoxic T cell-mediated damage to skin, gut, liver, and other organs. The chronic form of the disease is manifested much later (over 100 days post-BMT) and is characterized by hyperproduction of immunoglobulin (Ig), including autoantibodies, and damage to the skin, kidney, and other organs caused by Ig-deposition. The development of acute GVHD is predictive of the subsequent development of chronic GVHD. Thus, the same patient can develop both diseases, in sequence. Approximately 50% of all BMT patients develop either acute or chronic GVHD. Nearly 90% of acute GVHD patients go on to develop chronic GVHD. No current therapies for chronic GVHD are successful in the majority of patients.

GVHD can be modeled in the mouse using parental into F1 cell transplantation regimens. In the model described here, splenocytes from the DBA2 strain of mice are injected iv into (DBA2 x C57Bl/6) F1 mice, which are referred to as B6D2F1. The injected splenocytes constitute the graft, and the DBA2 mouse is the donor of that graft.

The F1 mouse which receives the graft is the host. Donor T cells present in the graft recognize half of the MHC markers (haplotypes) on host cells as foreign, because they are derived from the other, C57Bl/6 parent. This induces a donor T cell response against the host resulting in GVHD. When DBA/2 parental splenocytes are injected  
5 into the B6D2F1 host, chronic GVHD develops. In contrast when C57Bl/6 splenocytes are injected into the B6D2F1 host, acute GVHD develops. Although it remains unclear what underlying mechanism is responsible for the distinct disease outcomes using these 2 injection protocols it is believed that the cytokines expressed by the cells contained within the DBA/2 splenocyte graft favor the development of chronic GVHD while the  
10 cytokines expressed by the cells contained within the C57Bl/6 splenocyte graft favor the development of acute GVHD. Reagents which interfere with T cell interactions with antigen presenting cells (eg. dendritic cells, macrophages, B cells: APC) effectively block both acute and chronic GVHD.

A number of lines of evidence suggest that acute GVHD is a Th1 mediated  
15 disease (Krenger and Ferrara, Immunol. Res. 15: 50-73 (1996), Williamson et al., J. Immunol. 157: 689-699 (1996)). Cytotoxic activity by CD4+ and CD8+ T cells, by natural killer (NK) cells, and by activated granulocytes such as macrophages, is a well defined consequence of Th1 mediated T cell response, and shows a characteristic dependence on the expression of Il-2 and IFN- $\gamma$ , which are typical Th1 cytokines. Such  
20 cytotoxicity is a defining characteristic of acute GVHD. Furthermore, reagents which block critical cytokines involved in Th1 T cell differentiation, such as mAbs to Il-2 and Il-12, block the development of acute GVHD. Cytotoxicity can be directly cellular (eg. by phagocytosis of host cells) or by the action of secreted cytokines such as TNF which can induce apoptosis, or cell death. The consequence of donor anti-host cytotoxicity  
25 can be seen in a number of ways. First, host lymphocytes are rapidly destroyed, such that mice experiencing acute GVHD are profoundly immunosuppressed. Secondly, donor lymphocytes become engrafted and expand in the host spleen, and their cytotoxic activity can be directly measured in vitro by taking advantage of cell lines which express the host antigens that can be recognized (as foreign) by the donor cells. For  
30 example, cell lines expressing the appropriate antigens can be labeled with radioactive chromium51 isotope. Release of this radioactive isotope into the culture media is



evidence of the death of the labeled cell. Third, the disease becomes lethal as additional tissues and cell populations are destroyed, and therefore survivorship is a measurable consequence of disease.

Chronic GVHD appears to be a Th2 T cell mediated disease (De Wit et al., J. Immunol. 150: 361-366 (1993)). In the mouse model the development of the disease is dependent on the Th2 cytokine Il4, and can be blocked by treating with anti-Il4 mAb. Such treatment blocks the expansion of host B cells, and the concomitant hyper-Ig production. The development of GVHD can be followed in a number of ways. The expansion of the donor T cell and host B cell populations can be measured by the spleen index, which is the ratio of spleen weight to body weight, normalized to control (non-diseased) mice. The activation of B cells in diseased mice can be measured using analyses of B cell activation markers. Finally, the effects of B cell activation can be seen in the levels of Ig in circulation (eg. in serum) or produced by cultures of host splenocytes harvested several weeks after disease induction. Circulating Ig in diseased animals will contain anti-self antibodies. Ultimately, diseased animals succumb to kidney and other organ failure due to accumulated Ig deposition, and therefore survivorship is a relevant measure of disease activity.

We now show that a monoclonal antibody specific for TWEAK effectively and specifically blocks aspects of the development of GVHD, using the mouse model of chronic GVHD. The block in development of chronic GVHD is shown as a reduction in the spleen index, the loss of activation markers on host B cells, and reduced Ig production in the anti-TWEAK treated animals.

### SUMMARY OF THE INVENTION

The invention provides methods for blocking the development or treating or reducing the severity or effects of an immunological disorder in an animal including administering a pharmaceutical composition which comprises a therapeutically effective amount of a TWEAK blocking agent and a pharmaceutically acceptable carrier. The compound may be an antibody directed against a TWEAK ligand; an antibody directed against a TWEAK receptor; an agent that modifies the binding of the

TWEAK ligand to a TWEAK receptor; an agent that modifies cell surface receptor clustering; and an agent that can interrupt intracellular signaling of a TWEAK receptor. In a preferred embodiment the antibody is a monoclonal antibody. In a more preferred embodiment the monoclonal antibody is directed against the TWEAK surface ligand.

5 The animal may be mammalian and may be a human. The TWEAK blocking agent may be a soluble TWEAK receptor having a ligand binding domain that can selectively bind to a surface TWEAK ligand. In one embodiment the soluble TWEAK receptor may include a human immunoglobulin IgG domain. In a preferred embodiment, the human immunoglobulin IgG domain includes regions responsible for specific antigen  
10 binding.

The invention further includes a method for inhibiting an immune response in an animal, including administering a pharmaceutical composition which comprises an effective amount of a TWEAK blocking agent and a pharmaceutically effective carrier. The immune response may be Th1 or a Th2 cell-mediated immune response or both.

15 The invention also includes a composition having a therapeutically effective amount of a TWEAK blocking agent and a pharmaceutically acceptable carrier.

### **BRIEF DESCRIPTIONS OF THE DRAWINGS**

20 **Figure 1** shows a sequence alignment of soluble recombinant murine and human TWEAK proteins.

**Figure 2** shows a Fluorescent Activated Cell Sorting (FACS) analysis of the binding of hamster anti-TWEAK mAb AB.D3 to EBNA293 cells expressing murine TWEAK  
25 protein or human TWEAK protein, compared to the binding of a hamster mAb which recognizes an irrelevant protein (Keyhole Limpet Hemocyanin).

**Figure 3** shows a FACS analysis of the ability of mAb AB.D3 to block the binding of FLAG-tagged recombinant soluble human TWEAK to TWEAK receptor positive cells.

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**Figure 4** shows a FACS analysis of the activation state of B cells in mice undergoing chronic GVHD, treated with anti-TWEAK mAb AB.D3, anti-CD40L mAb MR1, control mAb, or untreated.

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### **DETAILED DESCRIPTION OF THE INVENTION:**

#### **Definitions**

10           In order that the invention herein described may be fully understood, the following detailed description is set forth.

          The terms "humoral response" and "cellular response" as used herein refer to the immunological response of an animal to an antigen whereby the animal produces antibodies to an antigen or produces a cytotoxic response to the antigen, or both. The  
15   Th1 class of T helper cells are important for the induction of the cellular response, and the Th2 class of T helper cells are important to the efficient production of high affinity antibodies.

          The term "T helper (Th) cells" as used herein, refers to a functional subclass of T cells which help to generate cytotoxic T cells and which cooperate with B cells to  
20   stimulate antibody production. Helper T cells recognize antigen in association with class II MHC molecules and provide contact dependent and contact independent (cytokine and chemokine) signals to effector cells.

          The term "Th1" refers to a subclass of T helper cells that produce TNF, interferon- $\gamma$  and IL-2 (and other cytokines) and which elicit inflammatory reactions  
25   associated with a cellular, i.e. non-immunoglobulin, response to a challenge.

          The term "Th2" refers to a subclass of T helper cells that produces IL-4, IL-5, IL-6, IL-10, and other cytokines, which are associated with an immunoglobulin (humoral) response to an immune challenge.

          The term "germinal center" as used herein refers to a secondary B cell follicle  
30   which forms after antigen immunization. The appearance of this histologic site

correlates with optimal memory generation, isotype switching, somatic hypermutation and thus the affinity maturation of an antibody response.

The term "antibody producing cells" refers to B cells which have received contact dependent and contact independent signals from Th cells, and are secreting immunoglobulins of the IgM, IgG, IgA, or IgE subclasses.

The term "Fc domain" of an antibody refers to a part of the molecule comprising the hinge, CH2 and CH3 domains, but lacking the antigen binding sites. The term is also meant to include the equivalent regions of an IgM or other antibody isotype.

The term "anti-TWEAK antibody" refers to any antibody that specifically binds to at least one epitope of the TWEAK protein.

The term "anti-TWEAK receptor antibody" refers to any antibody that specifically binds to at least one epitope of a TWEAK receptor.

The term "TWEAK receptor signaling" refers to molecular reactions associated with a TWEAK receptor pathway and subsequent molecular reactions which result therefrom.

The terms "TWEAK or TWEAK-receptor modifying agent" and "TWEAK or TWEAK-receptor modifying reagent" refers to any agent that can modify ligand binding to a TWEAK receptor, can modify cell surface TWEAK receptor clustering or TWEAK receptor signaling, or that can influence how a TWEAK receptor signal is interpreted within the cell.

The term "TWEAK ligand" or "TWEAK protein" refers to any TWEAK monomeric, polymeric, or heteromeric complex or derivative thereof that can specifically bind to a TWEAK receptor.

The term "subject" refers to an animal, or to one or more cells derived from an animal. Preferably, the animal is a mammal. Cells may be in any form, including but not limited to cells retained in tissue, cell clusters, immortalized, transfected or transformed cells, and cells derived from an animal that has been physically or phenotypically altered.

**TWEAK ligand**

TWEAK is a recently discovered member of the TNF family of proteins (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)). Members of the TNF family of proteins bind to receptors of the TNF-receptor (TNF-R) family of proteins. The interaction of proteins of the TNF family with their receptors influence a wide variety of functions within the immune system. Well known examples include the CD40L protein, which binds to the CD40 receptor to promote the differentiation of B cells into antibody producing cells (Grewal and Flavell, Immunol. Res. 16: 59-70 (1997)), the lymphotoxin-beta ligand (LT- $\beta$ ), which binds to the lymphotoxin-beta receptor to influence humoral immune responses by regulating the differentiation state of follicular dendritic cells (Mackay and Browning, Nature 395: 26-27 (1998)), and the OX40L, which binds the OX40 receptor to regulate the response of B cells to T cell signals (Flynn et al., J. Exp. Med. 188: 297-304 (1998)). Other ligand/receptor pairs within the TNF/TNF-R families which are known to play critical roles in the immune system include TNF/TNF-R55, FasL/Fas, and CD27/CD70.

TWEAK biology is as yet only partly understood. Purified soluble TWEAK protein was used to induce the differentiation and/or death of some tumor cell lines, including HT29 adenocarcinoma cells (cell death via apoptosis), HeLa cervical carcinoma cells (morphological changes), and A375 melanoma cells (anti-proliferation). TWEAK also induced the HT29 and A375 cell lines to secrete the chemokine IL-8, and had the same effect on a fibroblast cell line, WI-38 (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)). In addition TWEAK induced proliferation of a variety of normal endothelial cell lines (Lynch et al., J. Interferon Cytokine Res. 18: A-46 (1998)).

A putative receptor for TWEAK has been described (Marsters et al., Curr. Biol. 8: 525-528 (1998)) 1998). This receptor, variably known as TRAMP, Apo3, WSL-1, DR-3, or LARD is a member of the TNF-R family. Activation of TRAMP can induce apoptosis by engaging either the caspase-dependant cell death signaling pathway or cellular activation via NF- $\kappa$ B signaling pathways (Ashkenazi and Dixit, Science 281: 1305-1308 (1998)).

The expression of TWEAK in mouse and human tissues is widespread, with messenger RNA (mRNA) found in heart, brain, lung, liver, among other tissues, and



secondary lymphoid organs such as spleen, lymph nodes, and peripheral blood mononuclear cells (PBMCs). TWEAK does not appear to be expressed in the primary lymphoid organs where immune system cells develop, such as thymus, bone marrow, and fetal liver. Thus the roles that TWEAK may play in the immune system are likely  
5 be in immune responses, rather than in the development of the immune system.

The interaction of protein ligands and their receptors can be modified using a number of specific tools. For example, monoclonal antibodies (mAbs) which specifically recognize the ligand can be used to prevent or modify ligand/receptor binding, by virtue of recognition of the binding site, or by physically interfering with  
10 the interaction. Alternatively, anti-ligand mAbs can influence receptor signaling by influencing the binding of other ligands, when multiple ligands exist for a receptor. Such complicated effects have been noted for mAbs to the B7 family of CD28 ligands (Lenschow et al. J. Exp. Med. 181: 1145-1155 (1995)). Anti-ligand mAbs may have even more subtle effects in systems where multiple receptors exist for a particular  
15 ligand, eg. by modifying ligand binding to one receptor but leaving binding to another receptor unchanged. MABs which recognize the receptor can also be used to modify ligand binding, or may themselves induce or modify receptor signaling. Thus anti-receptor mAbs can be antagonistic or agonistic. Examples of mAbs used in the mouse or human systems to modify ligand/receptor interactions of TNF/TNF-R family  
20 members include mAbs specific for TNF, for CD40L, for LT- $\beta$ , for Fas-L, and for TR2/HVEM, among others. MABs can be used to block the initiation or development of immunologic and other diseases. For example, anti-CD40L has been used in treatment of systemic lupus erythematosus (SLE), and to control organ transplant failure.

25 Potent inhibitors of ligand/receptor interaction can also be created by cloning the sequences which encode the extracellular portion of receptor sequences to sequences which encode human immunoglobulin (Ig) heavy chain, then expressing the hybrid gene, using an appropriate gene promoter, in an appropriate cell line. Purified receptor-Ig fusion proteins can be used in vitro and in vivo to bind to available protein  
30 ligand, and thus modify the interaction of the ligand with the native, cell bound, receptor. Modification can occur by a variety of mechanisms, similar to those outlined

above for anti-ligand mAbs. Examples of receptor-Ig fusion proteins used in the mouse or human systems to modify ligand/receptor interactions of TNF/TNF-R family members include TNF-R55-Ig, TNF-R75-Ig, LT $\beta$ -R-Ig, and OX40-Ig, among others. Receptor-Ig fusion proteins can be used to block the initiation or development of immunologic and other diseases. For example, TNF-R75-Ig has been used to treat Inflammatory Bowel Disease (IBD).

Herein we demonstrate that a mAb which specifically binds to murine and human TWEAK blocks the development of a prototypical antigen-driven immunological disorder, Graft-Versus-Host Disease (GVHD). Our invention anticipates the use of TWEAK and TWEAK receptor(s) modifying reagents to treat a variety of immunological disorders which result from the introduction of foreign antigen into patients, such as GVHD which results from bone marrow or stem cell transplantation, and organ transplant failure resulting from graft rejection. Furthermore this invention anticipates the use of TWEAK and TWEAK receptor(s) modifying reagents to treat a variety of autoimmune disorders, such as SLE, Idiopathic Thrombocytopenia Purpura, Wegener's Granulomatosis, Polyarteritis Nodosa, Retinal Uveitis, Rapidly Progressive Crescentic Glomerulonephritis, Rheumatoid Arthritis, Multiple Sclerosis, and ulcerative colitis, among other examples. Other anticipated uses include treatment of acute and chronic inflammatory conditions, such as allergic inflammation, asthma, eosinophilia, Graves' disease, and Chagas' disease, among others.

### Materials and Methods

*Mice* Six to eight week old female mice of the DBA/2 and C57Bl/6 strains, and the (DBA/2 x C57Bl/6)F1 cross were purchased from Jackson Laboratory (Bar Harbor, ME USA), housed under conventional barrier protection, and handled in accordance with institutional guidelines.

*Monoclonal antibodies* Monoclonal antibodies which recognize human and murine TWEAK protein were generated in Armenian hamsters using soluble

human TWEAK protein that had been generated in baculovirus and purified as described (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)). For the first immunization each hamster received 50 $\mu$ gs TWEAK in complete Freund's adjuvant (CFA), injected ip. For the subsequent immunizations (days 14, 28, and 42 after the primary immunization) each hamster received 50 (days 14 and 28) or 33 (day 42)  $\mu$ gs TWEAK in Incomplete Freund's Adjuvant (IFA), ip. The final immunization before fusion of the spleen cell for hybridoma formation was with 100 $\mu$ gs TWEAK without adjuvant, ip. Hybridoma generation was performed using standard procedures (Lerner, Yale J. Biol. Med. 54: 387-402 (1981)).

The hybridoma which produces anti-murine CD40L mAb MR1 (Noelle et al., Proc. Natl. Acad. Sci. USA 89: 6550-6554 (1992)) was purchased from ATCC (Rockville, MD USA). The hybridoma which produces anti-KLH mAb Ha4/8 was obtained from Dr. Mendrick (Human Genome Sciences, Inc. Rockville, MD USA).

*ELISA analyses of mAb activity* Anti-human TWEAK mAbs were tested for their ability to bind to human and murine TWEAK in a variety of experimental formats. Several mAbs raised against human TWEAK protein also recognized the mouse TWEAK protein in an initial screening of mAb activity which was done using ELISA format assays. Murine TWEAK protein was generated in baculovirus using methods similar to those described for human TWEAK protein (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)). Purified human and murine TWEAK proteins were coated onto 96 well plates, and various hamster mAbs were tested for their ability to bind to these immobilized proteins. The capture of hamster mAb by the immobilized TWEAK proteins was visualized using Peroxidase-coupled donkey anti-hamster IgG (Jackson ImmunoResearch, West Grove, PA USA), and an appropriate peroxidase-dependent enzymatic reaction.

*FACS analyses of mAb activity* Soluble TWEAK protein induces apoptosis in HT29 cells, indicating that these cells express a TWEAK receptor (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)). Purified mAbs (10 $\mu$ g/ml) were tested for their ability to block the binding of FLAG-tagged murine or

human TWEAK to HT29 cells, as detected by a biotinylated anti-FLAG antibody, streptavidin-peroxidase, and appropriate enzymatic substrate.

*Induction of chronic GVHD* 6-8 week old DBA/2 and B6D2F1 female mice were sacrificed, and the spleens were removed using sterile technique. Single cell suspensions were made by gently grinding the organs between rough glass slides, allowing gross debris to settle out, then pelleting the cleared supernatant. This cell pellet was resuspended in Gey's buffered hypertonic solution, and incubated on ice for 3' to lyse red blood cells. 5 ml Geys was used per each spleen disrupted. The cell solution was pelleted again, resuspended in sterile, pyrogen-free PBS, pelleted, and resuspended a second time in sterile, pyrogen-free PBS. Cell number was determined using a hemocytometer, and the cell density was adjusted to  $2 \times 10^8/\text{ml}$ . This solution was then passed through a sterile 70um cell filter, and kept on ice until use. 6-8 week old B6D2F1 female mice were used as recipients. Each recipient was injected with 500 $\mu\text{l}$  ( $1 \times 10^8$ ) cells in the tail vein. Experimental groups received the DBA/2 graft (DBA/2 > F1), while a set of control animals received a B6D2F1 graft (F1 > F1). Animals were assayed for the development of chronic GVHD 14 days after injection.

*Blocking chronic GVHD* Mice received anti-TWEAK mAb AB.D3, anti-CD40L mAb MR1, control mAb Ha4/8, or no treatment. The treatment schedule was 250 $\mu\text{g}$ s mAb ip 4 hours prior to graft injection, and 2, 4, and 6 days afterwards.

*Assays of disease development* On day 14 of the experiment the mice were sacrificed and weighed. Then, the spleen was aseptically removed and weighed. The spleen index was calculated as the ratio of spleen to body weight for each animal in the control F1>F1 group, averaged to give a value of one. The average spleen index for all other groups is normalized to the control value. Splenocytes were then isolated from these spleens, as described above, then diluted to a concentration of  $1 \times 10^7$  cells/ml in sterile PBS. For FACS analysis 100  $\mu\text{l}$ s of cell were stained for the following cell surface markers. All mAbs described were purchased from Pharmingen (San Diego, CA. USA). The streptavidin reagent was purchased from Southern Biotechnology Corp

(Birmingham, AL., USA). Splenocytes were stained with biotinylated anti-H-2K<sup>b</sup> a haplotype marker which can distinguish the donor cells (H-2K<sup>b</sup> -) from host cells (H-2K<sup>b</sup> +) in various combinations with directly conjugated FITC or PE labelled anti-CD4, anti-CD8, anti-B220, anti-CD69, anti I-A<sup>d</sup>, anti-L-selectin, and anti-H2D<sup>d</sup>, in  
5 PBS/0.5%Bovine serum albumin (BSA)/0.1%sodium azide (FACS buffer) containing 10µg/ml FcBlock<sup>™</sup> (Pharmingen) to interrupt non-specific binding to Fc receptors. The cells were incubated with the mAbs for 1 h on ice. Each sample was then resuspended in 2 ml FACS buffer to wash, then centrifuged to pellet the cell. The cells were resuspended in FACS buffer containing CyChrome labelled streptavidin, which bound  
10 to the biotinylated mAb to provide a third channel for FACS analysis, washed a final time, and analyzed using FACScan instrumentation and Cellquest software (Becton Dickinson, San Jose, CA. USA).

For in vitro analysis of Ig secretion cells were pelleted and resuspended in DMEM containing 10% Fetal Bovine Serum (FBS)/4mM glutamine, at a concentration  
15 of  $1 \times 10^7$  cells/ml. One ml per well was distributed into 6 well plates. Cell supernatants were recovered after 24 hours.

**Source of Anti-TWEAK Antibodies** In one embodiment of this invention, antibodies (Abs) directed against TWEAK function as TWEAK blocking agents. Abs  
20 can be raised against monomeric, dimeric, or trimeric forms of the TWEAK protein, with or without heterologous subunits, if these exist. Furthermore, Abs can be raised against soluble, mutant, altered, or chimeric forms of TWEAK proteins. The anti-TWEAK Abs of this invention can be polyclonal or monoclonal (mAbs) and can be modified to optimize their ability to block TWEAK binding to its receptor(s), their in  
25 vivo bioavailability, stability, or other desired traits.

Polyclonal antibody sera directed against TWEAK are prepared using conventional techniques by injecting animals such as goats, rabbits, rats, hamsters or mice subcutaneously with human TWEAK in complete Freund's adjuvant (CFA), followed by booster intraperitoneal or subcutaneous injection in incomplete Freund's  
30 adjuvant (IFA). Polyclonal antisera containing the desired Abs directed against TWEAK are screened by conventional immunological procedures.



Hamster monoclonal antibodies (mAbs) directed against human TWEAK are prepared using conventional methods by injecting armenian hamsters subcutaneously with recombinant soluble human TWEAK in CFA, followed by booster intraperitoneal or subcutaneous injection in IFA. A hybridoma cell line (AB.D3.7.2) which produces the hamster anti-TWEAK mAb AB.D3 was deposited on ----- with the American Type Culture Collection (ATCC) (Rockville, MD) according to the provisions of the Budapest Treaty, and was assigned the ATCC accession number -----. All restrictions on the availability to the public of the above ATCC deposit will be irrevocably removed upon the granting of a patent on this application.

Various forms of anti-TWEAK Abs can also be made using standard recombinant DNA techniques (Winter and Milstein, Nature, 349, pp. 293-99 (1991)). For example, "chimeric" antibodies can be constructed in which the antigen binding domain from an animal antibody is linked to a human constant domain (e.g. Cabilly et al., US 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81, pp. 6851-55 (1984)). Chimeric Abs reduce the observed immunogenic responses elicited by animal Abs when used in human clinical treatments.

In addition, recombinant "humanized antibodies" which recognize TWEAK can be synthesized. Humanized Abs are chimeras comprising mostly human IgG sequences into which the regions responsible for specific antigen-binding have been inserted (e.g. WO 94/04679). Animals are immunized with the desired antigen, the corresponding Abs are isolated, and the portion of the variable region sequences responsible for specific antigen binding are removed. The animal-derived antigen binding regions are then cloned into the appropriate position of human antibody genes in which the antigen binding regions have been deleted. Humanized Abs minimize the use of heterologous (inter-species) sequences in human Abs, and are less likely to elicit immune responses in the treated subject.

Construction of different classes of recombinant anti-TWEAK Abs can also be accomplished by making chimeric or humanized Abs comprising the anti-TWEAK variable domains and human constant domains (CH1, CH2, CH3) isolated from different classes of immunoglobulins. For example, anti-TWEAK IgM Abs with increased antigen binding site valencies can be recombinantly produced by cloning the

antigen binding site into vectors carrying the human IgM heavy chain constant regions (Arulanandam et al., J. Exp. Med., 177, pp. 1439-50 (1993); Lane et al., Eur. J. Immunol., 22, pp. 2573-78 (1993); Traunecker et al., Nature, 339, pp. 68-70 (1989)).

5 In addition, standard recombinant DNA techniques can be used to alter the binding affinities of recombinant Abs with their antigens by altering amino acid residues in the vicinity of the antigen binding sites. The antigen binding affinity of a humanized Ab can be increased by mutagenesis based on molecular modeling (Queen et al., Proc. Natl. Acad. Sci. U.S.A., 86, pp. 10029-33 (1989); WO 94/04679).

10 It may be desirable to increase or to decrease the affinity of anti-TWEAK Abs for TWEAK depending on the targeted tissue type or the particular treatment schedule envisioned. For example, it may be advantageous to treat a patient with constant levels of anti-TWEAK Abs with reduced ability to modify the TWEAK pathway for semi-prophylactic treatments. Likewise, anti-TWEAK Abs with increased affinity for TWEAK may be advantageous for short-term treatments.

15 The production of hamster anti-human TWEAK monoclonal antibody is illustrated in EXAMPLE 1.

**Use of Anti-TWEAK mAb to Block the Development of Chronic GVHD, an Antigen-Driven Immunological Disease.** We now show the effects of a TWEAK  
20 blocking agent, mAb AB.D3, on the development of an immunological response in a mouse model of chronic graft-versus-host disease (GVHD). The ability to block chronic GVHD includes effects on B cell activation and proliferation, and on the generation of secreted IgG. Mice were treated intraperitoneally (ip) with 250µgs anti-TWEAK mAb AB.D3, anti-KLH control mAb Ha4/8, anti-CD40L mAb MR1, or were  
25 left untreated. 4 hours later mice received  $1 \times 10^8$  splenocytes isolated from DBA/2 mice, in an 0.5ml injection given intravenously (iv). The iv injected DBA/2 splenocytes constituted the allograft. 2, 4, and 6 days after the graft was given, the mice were again treated with 250µgs anti-TWEAK mAb AB.D3, anti-KLH control mAb Ha4/8, or anti-CD40L mAb MR1. A control group of mice received  $1 \times 10^8$   
30 B6D2F1 splenocytes, which cannot induce disease in B6D2F1 recipients.

Alternatively, ungrafted and untreated B6D2F1 mice were used as controls. 14 days after the graft was given the mice were sacrificed and examined for evidence of disease.

Untreated graft-recipient mice manifest a variety of symptoms that are indicative of the development of chronic GVHD. Splenomegaly, or enlargement of the spleen, is evidence that donor T cells and host B cells have become activated, and are undergoing polyclonal expansion, with dramatic increases in cell number. The appearance of cell surface proteins such as CD69 on a subset of B cells is indicative of B cell activation. The loss of L-selectin molecules from CD4+ and CD8+ T cells is evidence of T cell activation. The secretion of Ig molecules, such as IgG classes, IgA, and IgE, either into the serum, or in in vitro cell culture assays, indicates that B cells have become activated, and have switched their Ig class. In this regard the appearance of anti-self Igs in the serum or in in vitro cell culture assays shows that Igs that are being produced have inappropriate autoantigen recognition. Finally, survivorship can be measured as an outcome of different treatment regimens.

We compared control mice to untreated allograft-recipient mice to examine the extent of splenomegaly, B cell activation, and Ig secretion during GVHD. Anti-CD40L mAb MR1 was used as a positive control in these experiments, since it has been previously shown that blocking the CD40L/CD40 interaction is an effective means of interfering with the development of chronic GVHD (Durie, et al., J. Clin. Invest. 94: 1333-1338 (1994)). A hamster mAb, Ha4/8, raised to Keyhole Limpet Hemocyanin (KLH) served as a negative control treatment. Results from 2 experiments are shown in Table 2. In both experiments treatment with anti-TWEAK mAb AB.D3 reduced the amount of splenomegaly (compared to untreated allograft-recipients) approximately 33%. Treatment with the negative control mAb Ha4/8 had no effect, while treatment with anti-CD40L mAb MR1 blocked splenomegaly by nearly 70%. To investigate cell populations affected by treatment with anti-TWEAK mAb AB.D3 FACS analyses were performed on splenocytes taken from the recipient mice 14 days after graft injection. Spleen cells from 3-4 mice per group were isolated and pooled. Activation of recipient B cells is a defining feature of chronic GVHD. In untreated and control mAb treated mice a small but readily visible proportion of the B200+ B cells express the activation marker CD69 (Figure 4 and Table 3). In contrast, virtually no B200+ B cells in MR1 or

AD.B3 treated mice express CD69. Lack of measurable B cell activation in the spleen of AB.D3 mice could be due to one or more of several mechanisms of action, including failure of T cell activation, failure of B cell activation, or cell death (apoptosis). Next we measured total IgG in cultures of splenocytes in mice from different treatment groups to determine if the loss of the CD69 activation marker from the B cell populations correlated with a functional readout, ie. IgG production. Untreated control mice, MR1 treated mice, and AB.D3 treated mice produced dramatically lower amounts of total IgGs than did untreated or Ha4/8-treated allograft-recipient mice (Table 4). This result shows that Ig secretion by activated B cells, a defining feature of chronic GVHD, is blocked by treatment with anti-TWEAK mAb. The use of anti-TWEAK mAb to block the development of chronic GVHD is illustrated in Example 2.

**Other Antibody Mediated Diseases** Many organ-specific and systemic autoimmune diseases involve pathological antibody responses. Such conditions include: Myasthenia Gravis, autoimmune hemolytic anemia, Chaga's disease, Grave's disease, idiopathic thrombocytopenia purpura (ITP) Systemic Lupus Erythematosus (SLE), Wegener's Granulomatosis, Poly-arteritis Nodosa and Rapidly Progressive Crescentic Glomerulonephritis (From Benjamini, et al. Immunology, A Short Course, (Wiley-Liss, New York 3d ed. (1996))

Although the etiology of SLE is undefined, a fair amount is known about the immunologic mechanism responsible for the pathology observed. For unknown reasons, patients with SLE produce antibodies against nuclear components of the body (antinuclear antibodies (ANA) notably against native double stranded DNA. Clinically the presence of these antibodies correlates best with the renal pathology that develops in SLE. These antibodies complex with DNA apparently derived from the breakdown of normal tissue, and as in any immune - aggregate disease, such complexes form deposits trapped against the basement membrane of the glomeruli, in arteriolar walls and in joint synovial spaces. These complexes activate the complement cascade and attract granulocytes. The subsequent inflammatory reaction is characterized as glomerulonephritis, with resulting damage to the kidneys leading to proteinuria and hematuria.

SLE has been studied in murine models for decades. The development of SLE can be blocked using reagents such as CTLA4-Ig fusion protein and anti-CD40L, which interrupt critical steps in T and B cell activation (Grewal and Flavell, Immunol. Rev. 153: 85-106 (1996)). Recently, the therapeutic efficacy of a reagent specific for the murine CD40 ligand was evaluated in several models (Mohan, et al., J. Immunol., 154, pp. 1470-1480 (1995)). The acceleration of lupus by the transfer of cells which induce the production of pathogenic antibodies in vivo was shown to be inhibited by administration of a monoclonal antibody which blocks CD40/CD40 ligand interactions. Moreover a brief treatment of lupus mice with anti-CD40 ligand antibody had a sustained beneficial effect on their spontaneous disease long after the antibody had been cleared from their systems. The experimentation indicated that pathogenic B cells could not produce antibody even 9 months after the therapy suggesting that there was a delay of the expansion of autoimmune memory B cells resulting in long-term therapeutic benefits. Moreover, anti CD40L treatment was able to halt or delay the progression of established SLE in a spontaneously occurring mouse model (Kalled, et al., J. Immunol. 160: 2158-2165 (1998)). As we have shown that reagents which modify TWEAK/TWEAK receptor(s) interaction in vivo inhibit B cell activation and Ig production, reagents of this invention will be useful for treating or preventing SLE.

The normal immune response to some pathogenic infectious agents elicits autoantibody responses that can become excessive and present a medical problem. One example is Chagas' disease, an inflammatory cardiomyopathy which develops in humans and experimental animals with chronic *Trypanosoma cruzi* (T. cruzi) infection. Recently, several studies have identified anti-self antibodies in the sera of Chagas' disease patients (Bach-Elias et al., Parasitol. Res. 84: 796-799 (1998)). Furthermore, induction of heart - specific autoimmune responses has recently received substantial experimental support as a possible mechanism involved in the pathogenesis of human Chagas' cardiomyopathy. A recent study (Tibbetts, et al., J. Immunol., 152, pp. 1493 - 1499 (1994)) determined that cardiac antigen-specific antibodies are produced in T. Cruzi - infected C57Bl/6 mice with heart disease. Upon infection with the Brazil strain of T. Cruzi, C57Bl/6 mice develop a cardiomyopathy that is histologically similar to that observed in chronically infected humans. Antisera from these mice react with three



cardiac antigens while C57Bl/6 mice infected with the Guayas strain of T. Cruzi which do not develop cardiomyopathy did not produce such antibodies. These data indicate that these antibodies are specific markers of cardiomyopathy. The ability of TWEAK modifying agents to block B cell activation and Ig production will be useful in blocking the cardiac damage that occurs in Chagas' disease patients.

Another example of cell destruction by autoantibodies generated as a consequence of certain infectious diseases or for other unknown reasons is idiopathic thrombocytopenia purpura (ITP). In this condition antibodies directed to platelets result in platelet destruction (by complement or phagocytic cells with Fc or C3b receptor) which may lead to bleeding. Therapeutics which will inhibit such antibody mediated autoimmune reactions in vivo such as the TWEAK modifying agents of this invention - which inhibit antibody generation -- will be useful to treat or prevent these autoimmune diseases as well.

The normal immune response to some pathogenic infectious agents can also elicit hypersensitivity reactions that can become excessive and present themselves as a medical problem. The most prevalent example of type I hypersensitivity is allergic reaction. These are mediated by IgE antibodies which bind via their Fc portion to receptors on mast cells and basophils to trigger the release of pharmacologically active agents that mediate anaphylaxis. ITP and Goodpasture's syndrome are sometimes thought to be Type II reactions which occur when IgM or IgG antibodies bind to antigen on the cell surface and activate the complement cascade. Granulocytes are then attracted to the site of activation, and damage from the release of lytic enzymes from their granules results in the destruction of cells. Rheumatic arthritis is thought to result from a type III hypersensitivity reaction mediated by immune complexes of antigen (in this case rheumatoid factor, an IgM autoantibody) that binds to the Fc portion of normal IgG. These immune complexes participate in causing inflammation of joints and the damage characteristic of this disease. As these pathologies are mediated in part by antibodies, therapeutics which will inhibit the generation of antibody, such as the TWEAK modifying agents of this invention, will be useful for treating or preventing these diseases as well.

Additional examples of diseases which cause significant antibody-mediated damage to the patient include Graves' disease and acute hemolytic anemia. It is anticipated that TWEAK modifying agents will show efficacy in preventing or treating such diseases

5

**Inhibition of Cellular Immune Responses** We have demonstrated that administration of reagents which modify the activity of TWEAK protein in vivo block the development of an antibody-mediated (humoral) immune disorder, chronic GVHD. Other potent immune system modifiers which are capable of blocking the development of chronic GVHD include anti-CD40L and CTLA4-Ig fusion protein. These reagents block critical steps in T and B cell activation (Grewal and Flavell, Immunol. Rev. 153: 85-106 (1996)). Therefore, therapeutic manipulation of these other potent immune system pathways has not been limited to the humoral immune responses. For example anti-CD40L and CTLA4-Ig have been used separately and together to control organ transplant rejection in animal models (Kirk et al., Proc. Natl. Acad. Sci. USA. 94 :8789-8794 (1997)). Much of the immune response to transplanted organs is Th1 T cell mediated, and consists of a cytotoxic cellular immune response. Such cellular immune responses are responsible for cell mediated damage in a variety of other immune disorders such as autoimmune diseases such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, diabetes, ulcerative colitis, and Crohn's disease, as examples. Based on the ability to block the development of chronic GVHD, our invention anticipates the use of TWEAK or TWEAK-receptor modifying reagents in the therapeutic treatment of cellular immune disorders.

**Treatments Using TWEAK and TWEAK-Receptor Modifying Agents** The compositions of this invention will be administered at an effective dose to treat the particular clinical condition addressed. Determination of a preferred pharmaceutical formulation and a therapeutically efficient dose regiment for a given application is well within the skill of the art taking into consideration, for example, the condition and weight of the patient, the extent of desired treatment and the tolerance of the patient for the treatment.

Doses of about 5 mg/kg of a TWEAK or TWEAK-Receptor modifying agent are expected to be suitable starting points for optimizing treatment doses.

Determination of a therapeutically effective dose can also be assessed by performing in vitro experiments that measure the concentration of the modifying agent required to coat target cells (TWEAK or TWEAK-Receptor-positive cells depending on the modifying agent) for suitable (therapeutic) time periods. The FACS and ELISA receptor-ligand binding assays described herein can be used to monitor the cell coating reaction. Based on the results of such in vitro binding assays, a range of suitable modifying agent concentrations can be selected to test in animals.

Administration of the soluble modifying agents of this invention, alone or in combination, including isolated and purified forms of anti-TWEAK and anti-TWEAK-R antibodies, receptor-Ig fusion proteins, other TWEAK and TWEAK-receptor modifying reagents including naturally occurring or chemically derived reagents, and their salts or pharmaceutically acceptable derivatives thereof, may be accomplished using any of the conventionally accepted modes of administration of agents which exhibit immunosuppressive activity.

The pharmaceutical compositions used in these therapies may also be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, suppositories, and injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. Modes of administration may include oral, parenteral, subcutaneous, intravenous, intralesional or topical administration.

The TWEAK and TWEAK-receptor modifying agents of this invention may, for example, be placed into sterile, isotonic formulations with or without cofactors which stimulate uptake or stability. The formulation is preferably liquid, or may be lyophilized powder. For example, the TWEAK and TWEAK-receptor modifying agents of this invention may be diluted with a formulation buffer comprising 5.0 mg/ml citric acid monohydrate, 2.7 mg/ml trisodium citrate, 41 mg/ml mannitol, 1 mg/ml glycine and 1 mg/ml polysorbate 20. This solution can be lyophilized, stored under refrigeration and reconstituted prior to administration with sterile Water-For-Injection (USP).

The compositions also will preferably include conventionally pharmaceutically acceptable carriers well known in the art (see for example Remington's Pharmaceutical Sciences, 16th Edition, 1980, Mac Publishing Company). Such pharmaceutically acceptable carriers may include other medicinal agents, carriers, genetic carriers, 5 adjuvants, excipients, etc., such as human serum albumin or plasma preparations. The compositions are preferably in the form of a unit dose and will usually be administered one or more times a day.

The pharmaceutical compositions of this invention may also be administered using microspheres, liposomes, other microparticulate delivery systems or sustained 10 release formulations placed in, near, or otherwise in communication with affected tissues or the bloodstream. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shaped articles such as suppositories or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (U.S. Patent No. 3,773,319; EP 58,481), copolymers of L-glutamic acid 15 and ethyl-L-glutamate (Sidman et al., Biopolymers, 22, pp. 547-56 (1985)); poly(2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al., J. Biomed. Mater. Res., 15, pp. 167-277 (1981); Langer, Chem. Tech., 12, pp. 98-105 (1982)).

**Advantages of Therapeutic Compositions Comprising TWEAK or**  
20 **TWEAK-Receptor Modifying agents** The TWEAK and TWEAK-receptor modifying agents of this invention are capable of inhibiting immune responses, as shown by the inhibition of B cell activation and Ig production in the chronic GVHD model. The ability to selectively inhibit such immune mediated responses will be useful for treating immune disorders including various autoimmune diseases, organ transplant rejection, 25 and acute and chronic inflammatory conditions. Treatment of such pathologic immune disorders generally employs immunomodulatory and immunosuppressive agents which have pleiotropic effects on a wide variety of cell types and immunological responses. These non-specific immunosuppressive agents are generally required in high and often cytotoxic doses that cause adverse side effects. For example, three general 30 immunosuppressive agents currently used include steroids, cyclophosphamide and azathioprine. Steroids are pleiotropic anti-inflammatory agents which suppress

activated macrophages and inhibit the activity of antigen presenting cells in ways which reverse many pathologic T cell effects. Cyclophosphamide, an alkylating agent, mediates cell death by inhibiting DNA replication and repair. Azathioprine is an anti-proliferative agent which inhibits DNA synthesis. These non-specific

5 immunosuppressive agents are generally required in high doses which increase their toxicity (e.g. nephro- and hepatotoxicity) and cause adverse side effects. They are thus unsuitable for long term therapies.

Thus, there is an unmet need for additional agents and therapies which overcome the problems caused by conventional treatments.

10 The following are examples which illustrate the anti-TWEAK mAb of this invention, the methods used in the characterization of the mAb, and the use of the mAb to block an antigen-driven immunological disorder. These examples should not be construed as limiting: the examples are included for purposes of illustration and the present invention is limited only by the claims.

15

### EXAMPLES

#### **EXAMPLE 1: Production of a monoclonal antibody to TWEAK protein.**

Monoclonal antibodies which recognizes human and murine TWEAK protein  
20 were generated in Armenian hamsters using soluble human TWEAK protein that had been generated in baculovirus as described (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)). For the first immunization each hamster received 50µgs TWEAK in complete Freund's adjuvant (CFA), injected ip. For the subsequent immunizations (days 14, 28, and 42 after the primary immunization) each hamster  
25 recieved 50 (days 14 and 28) or 33 (day 42) µgs TWEAK in Incomplete Freund's Adjuvant (IFA), ip. The final immunization before fusion of the spleen cell for hybridoma formation was with 100µgs TWEAK without adjuvant, ip. Hybridoma generation was performed using standard procedures (Lerner, Yale J. Biol. Med. 54: 387-402 (1981)).

30 Initial screening of mAb activity was done using ELISA format assays. Murine TWEAK protein was generated in baculovirus using methods similar to those described for human TWEAK protein (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410



(1997)). Purified human and murine TWEAK proteins were coated onto 96 well plates, and various hamster mAbs were tested for their ability to bind to these immobilized proteins. The capture of hamster mAb by the immobilized TWEAK proteins was visualized using HRP-coupled donkey anti-hamster IgG (Jackson ImmunoResearch, West Grove, PA USA), and an appropriate enzymatic reaction. Eight out of 23 mAbs recognized both murine and human TWEAK proteins (Table 1).

Table 1

10

CLONE	Human TWEAK ELISA	Murine TWEAK ELISA	Ligand Blocking FACS	FACS Binding to hTWEAK/293	FACS Binding to mTWEAK/293
AB.D3.7.2	+++	+++	+	+	+
AA.DB5	+++	-	+/-	+	-
AC.H5.24	+++	+++	-	nd	nd
AA.G9	+++	-	+/-	+	-
AB.H12.1	+++	+++	-	-	-
BA.F5.35.2 *	+++	+++	+	+	-
BG.A12.5 *	++	+++	+	+	-
BE.D5	++	-	+/-	+	-
AF.D4	++	-	+	+	-
AB.G11.1 *	+++	+++	+	+	+
BE.B3.6.1 *	++	-	-	+	-
AA.AC2.3.2	+++	-	+/-	nd	nd
BB.B12.1.31.3	++	-	+/-	+	-
AA.DG7.14 *	+++	+++	+	+	+
BD.A3	++	-	-	-	-
BC.B10.21 *	+++	+++	+	+	+
BG.E8	+++	-	+/-	+	-
AE.C10.4 *	+++	-	+	+	-
AA.BB4.2 *	+++	-	+	+	-
AA.EC10.2 *	+++	-	+	+	-
AD.B5.9 *	+++	-	-	-	-
AB.D4.67.19	+++	-	-	-	-

**HUMAN TWEAK ELISA:** mAbs were evaluated on their ability to bind to a human TWEAK coated plate.

15 **MURINE TWEAK ELISA:** mAbs were tested for their crossreactivity on a murine TWEAK coated plate.

**LIGAND BLOCKING FACS:** mAbs were tested as to whether they were able to block human TWEAK-flag binding to HT29 cells.

20 **FACS ANALYSIS OF EBNA293 TRANSFECTANTS:** Human and murine TWEAK cDNAs were used to transfect EBNA293 cells, and mAbs were tested for reactivity 3 days later

The conservation of antigenic epitopes across species lines was unsurprising given the extent of protein homology seen between the murine and human forms of TWEAK (Figure 1). FACS analyses were used to determine if anti-TWEAK mAbs could bind to TWEAK proteins expressed on the surface of a cell. Human and murine TWEAK cDNA sequences were cloned into the expression vector CH269 (Chicheportiche et al., J. Biol. Chem. 51: 32401-32410 (1997)), and these constructs were used in transfections to produce transient protein expression in EBNA 293 cells. Four mAbs, including AB.D3, bound well to EBNA293 cells expressing murine TWEAK (Figure 2). These 4 mAbs were also capable of preventing the binding of human TWEAK to HT29 cells which are known to express one or more TWEAK-receptors (eg., Figure 3). Together these FACS analyses indicated that anti-TWEAK mAbs specifically recognized TWEAK proteins, and were capable of modifying the ability of TWEAK proteins to bind to one or more TWEAK receptors.

#### EXAMPLE 2:

**The use of anti-TWEAK mAb AB.D3 to block the development of splenomegaly, activated B cells, and Ig production in a murine model of chronic GVHD.**

Chronic GVHD was induced in 6-8 week old B6D2F1 female mice using DBA/2 splenocyte grafts as described in methods. Each recipient was injected with 500 $\mu$ l ( $1 \times 10^8$ ) cells in the tail vein. Experimental groups recieved the DBA/2 graft (DBA/2>F1), while a set of control animals recieved a B6D2F1 graft (F1>F1). Mice received anti-TWEAK mAb AB.D3, anti-CD40L mAb MR1, control mAb Ha4/8, or no treatment. Animals were dosed with 250 $\mu$ g mAb ip 4 hours prior to graft injection, and 2, 4, and 6 days afterwards. On day 14 of the experiment the mice were sacrificed and the spleen index was calculated as the ratio of spleen weight to body weight for each animal in the control F1>F1 group, averaged

to give a value of one. The average spleen index for all other groups is normalized to the control value. The results from 2 independent experiments are shown in Table 2. Animals receiving the DBA/2 graft (DBA/2 > F1), and left untreated or treated with control mAb Ha4/8 showed a dramatic increase in spleen weight when compared to F1 > F1 graft controls. This result is reflected in the spleen index, which showed an increase from the normalized control value of 1.0 to 2.6. Treatment of mice with anti-CD40L mAb MR1 reduced the spleen index nearly to control levels (1.1), and treatment with anti-TWEAK mAb AB.D3 reduced splenomegaly by 35% to 1.7 (Table 2).

10 Table 2

<u>TREATMENT</u>	<u>EXPERIMENT 1</u>	<u>EXPERIMENT 2</u>	<u>AVERAGE</u>
F1 > F1 (control)	1.0	1.0	1.0
DBA/2 > F1, untreated	2.7	2.6	2.65
15 DBA/2 > F1, Ha4/8 treated	2.9	2.6	2.75
DBA/2 > F1, MR1 treated	1.1	1.1	1.1
DBA/2 > F1, AB.D3 treated	1.8	1.7	1.75

20 On day 14 of the experiment the mice were sacrificed and weighed. Then, the spleen was aseptically removed and weighed. The spleen index was calculated as the ratio of spleen to body weight for each animal in the control F1>F1 group, averaged to give a value of one. The average spleen index for all other groups is normalized to the control value.

25 FACS analyses were employed to analyze the activation state of lymphocyte populations in the spleens of control and diseased mice, representing the various treatment

groups. Initially we analyzed the amount of donor cell engraftment in the various group of mice. Donor cells were detected in the spleens of host mice at approximately the same percentage (average = 6.2%) in all DBA/2 > F1 treatment groups (Table 3). This result indicated that the different mAb treatments were not affecting donor cell engraftment.

5

Table 3

<u>TREATMENT</u>	<u>% H-2K<sup>b</sup> negative (donor) cells</u>
F1 > F1 (control)	0.2
10 DBA/2 > F1, untreated	5.9
DBA/2 > F1, Ha4/8 treated	9.9
DBA/2 > F1, MR1 treated	5.5
DBA/2 > F1, AB.D3 treated	4.3
15 For FACS analysis splenocytes from each group of mice were stained on ice for 1 h with PE-coupled anti-H2k <sup>b</sup> and FITC-coupled anti-H-2D <sup>d</sup> to measure expression of MHC alleles. Host splenocytes are positive for both markers, but donor splenocytes are H-2k <sup>b</sup> negative. Percent values reflect events within the lymphocyte population, as determined by forward and side scatter characteristics.	

20

Next, two markers of lymphocyte activation were employed: CD69 expression on B220+ B cells, and L-selectin expression of CD4+ and CD8+ T cells. When control and untreated DBA/2 > F1 mice were compared we noted an increase in CD69+ B cells from 2.5% to 6.2% (Table 4). This increase in CD69+ B cell numbers was prevented by

25 treatment with either MR1 (3.3%) or AB.D3 (2.1%) but not Ha4/8 (6.5%) (Figure 4 and Table 3). Data from 2 experiments are shown in Table 4.

Table 4

<u>TREATMENT GROUP</u>		<u>%B220+/CD69+ (double positive) CELLS</u>		
		<u>EXPERIMENT 1</u>	<u>EXPERIMENT 2</u>	<u>AVERAGE</u>
5	F1 > F1, control	2.5	2.0	2.25
	DBA/2 > F1, untreated	6.2	5.5	5.9
	DBA/2 > F1, Ha4/8 treated	6.5	4.7	5.6
	DBA/2 > F1, MR1 treated	3.3	nd	3.3
10	DBA/2 > F1. AB.D3 treated	2.1	3.2	2.65

15 Splenocytes were stained with PE-labelled anti-B220 and FITC labelled anti-CD69 for 1 h on ice, washed with FACS buffer, then analyzed. Percentages are based events collected within the lymphocyte population, as determined by forward and side scatter characteristics.

For in vitro analysis of Ig secretion ELISA analysis was performed on splenocyte cell culture supernatants derived from spleens harvested from the various treatment groups.

20 Results in Table 5 are from the second experiment, average of results from 3 mice per group. Treatment with MR1 or AB.D3 prevented the 4 - 6 fold increase in spontaneous IgG production observed in the untreated and Ha4/8 treated DBA/2 > F1 groups.

Table 5

25



<u>TREATMENT GROUP</u>		<u>SPONTANEOUS IN VITRO IgG PRODUCTION</u>
	F1 > F1, control	125ng/ml +/- 20ng/ml
	DBA/2 > F1, untreated	750ng/ml +/- 65ng/ml
5	DBA/2 > F1, Ha4/8 treated	500ng/ml +/- 150ng/ml
	DBA/2 > F1, MR1 treated	0
	DBA/2 > F1. AB.D3 treated	125ng/ml +/- 20ng/ml
<hr/>		
10	For in vitro analysis of Ig secretion cells were pelleted and resuspended in DMEM containing 10% Fetal Bovine Serum (FBS)/4mM glutamine, at a concentration of $1 \times 10^7$ cells/ml. One ml per well was distributed into 6 well plates. Cell supernatants were recovered after 24 hours. Values represent the average of 2 wells per mouse, with 3 mice represented for each treatment group.	

**CLAIMS**

What is claimed is:

1. A method for blocking the development or treating or reducing the severity or effects of an immunological disorder in an animal comprising the step of administering a pharmaceutical composition which comprises a therapeutically effective amount of a TWEAK blocking agent and a pharmaceutically acceptable carrier.
2. A method for inhibiting an immune response in an animal comprising the step of administering a pharmaceutical composition which comprises an effective amount of a TWEAK blocking agent and a pharmaceutically effective carrier.
3. The method according to claim 1 or 2, wherein the TWEAK blocking agent is selected from the group consisting of:
  - (a) an antibody directed against the TWEAK ligand;
  - (b) an antibody directed against the TWEAK receptor;
  - (c) an agent that modifies the binding of the TWEAK ligand to the receptor;
  - (d) an agent that modifies the cell surface receptor clustering; and
  - (e) an agent that can interrupt the intra cellular signaling of the TWEAK receptor.
4. The method according to claim 1 or 2, wherein the animal is mammalian.
5. The method according to claim 4, wherein the mammal is human.

6. The method according to claim 1 or 2, wherein the TWEAK blocking agent comprises a soluble TWEAK receptor having a ligand binding domain that can selectively bind to a surface TWEAK ligand.

5 7. The method of claim 6, wherein the soluble TWEAK receptor comprises a human immunoglobulin IgG domain.

8. The method of claim 7, wherein the human immunoglobulin IgG domain comprises regions responsible for specific antigen binding.

10

9. The method according to claim 1 or 2, wherein the antibody directed against the TWEAK receptor comprises a monoclonal antibody.

10. The method according to claim 1 or 2, wherein the TWEAK blocking agent comprises a monoclonal antibody directed against the TWEAK surface ligand.

15

11. The method according to claim 10, wherein the antibody is directed against a subunit of the TWEAK ligand.

20

12. The method according to claim 2, wherein the immune response is a Th1 cell-mediated immune response.

13. The method according to claim 2, wherein the immune response is a Th2 cell-mediated immune response.

25

14. The method according to claim 2, wherein the immune response includes both a Th1 and a Th2 cell-mediated immune response.

30

15. The method according to claim 2, wherein the TWEAK blocking agent comprises a monoclonal antibody directed against the TWEAK receptor.

16. A pharmaceutical composition comprising a therapeutically effective amount of a TWEAK blocking agent and a pharmaceutically acceptable carrier.

5 17. The composition according to claim 16, wherein the TWEAK blocking agent is selected from the group consisting of:

- (a) an antibody directed against the TWEAK ligand;
- (b) an antibody directed against the TWEAK receptor;
- 10 (c) an agent that modifies the binding of the TWEAK ligand to the receptor;
- (d) an agent that modifies the cell surface receptor clustering; and
- (e) an agent that can interrupt the intracellular signaling of the TWEAK receptor

15 18. The composition according to claim 16, wherein the TWEAK blocking agent comprises a soluble TWEAK receptor having a ligand binding domain that can selectively bind to a surface TWEAK ligand.

20 19. The composition according to claim 18, wherein the soluble TWEAK receptor comprises a human immunoglobulin IgG domain into which regions responsible for specific antigen binding have been inserted.

25 20. The composition of claim 16, wherein the TWEAK blocking agent comprises a monoclonal antibody directed against the TWEAK receptor.

21. The composition according to claim 16, wherein the TWEAK blocking agent comprises a monoclonal antibody directed against the TWEAK surface ligand.

30 22. The composition according to claim 21, wherein the antibody is directed against a subunit of the TWEAK ligand.

Human	1	MAARRSQRRRGRGEPGTALLVPLALGLGLALACLGLLLVVSLGSRASL
Mouse	1	-----VLSGLALACLGLLLVVSLGSWATL
Human	51	SAQEPAQEELVAEEDQDPSELNPQTEESQDPAPFLNRLVRPRRSAPKGRK
Mouse	27	SAQEPSQEELTAEDRREPPELNPQTEESQDVVPFLEQLVRPRRSAPKGRK
Human	101	TRARRATAAHYEVHPRPGQDGAQAGVDGTVSGWEEARINSSPLRYNRQI
Mouse	77	ARPRRAIAAHYEVHPRPGQDGAQAGVDGTVSGWEEKINSSPLRYDRQI
Human	151	GEFIVTRAGLYLYCQVHFDEGKAVYKLDLLVDGVLALRCLEEFSAATAA
Mouse	127	GEFTVIRAGLYLYCQVHFDEGKAVYKLDLLVNGVLALRCLEEFSAATAA
Human	201	SSLGPQLRLCQVSGLLALRPGSSLRIRTLPSWAHLKAAPFLTYFGLFQVH
Mouse	177	SSPGPQLRLCQVSGLLPLRPGSSLRIRTLPSWAHLKAAPFLTYFGLFQVH

FIG. 1



2/5

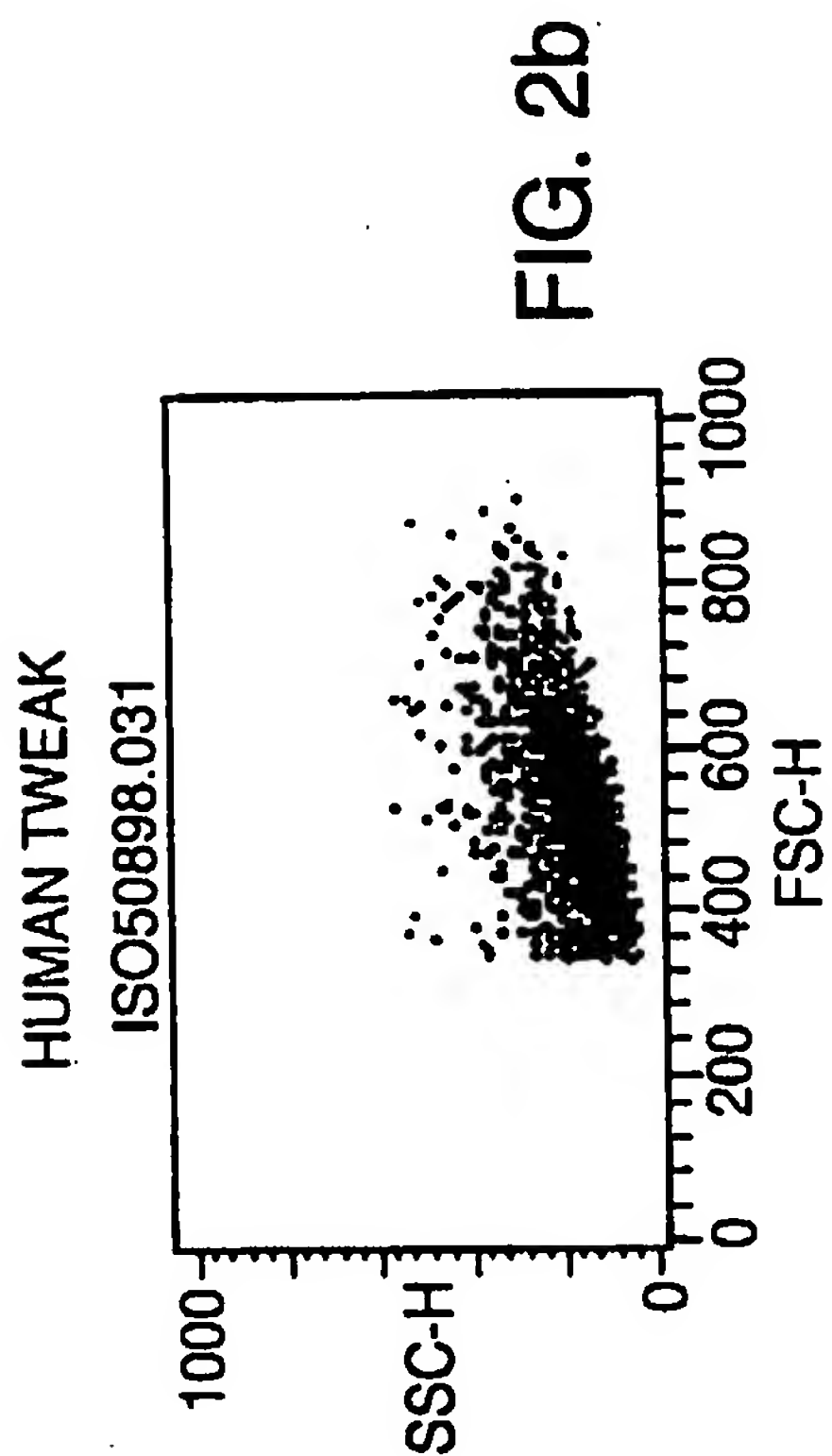


FIG. 2b

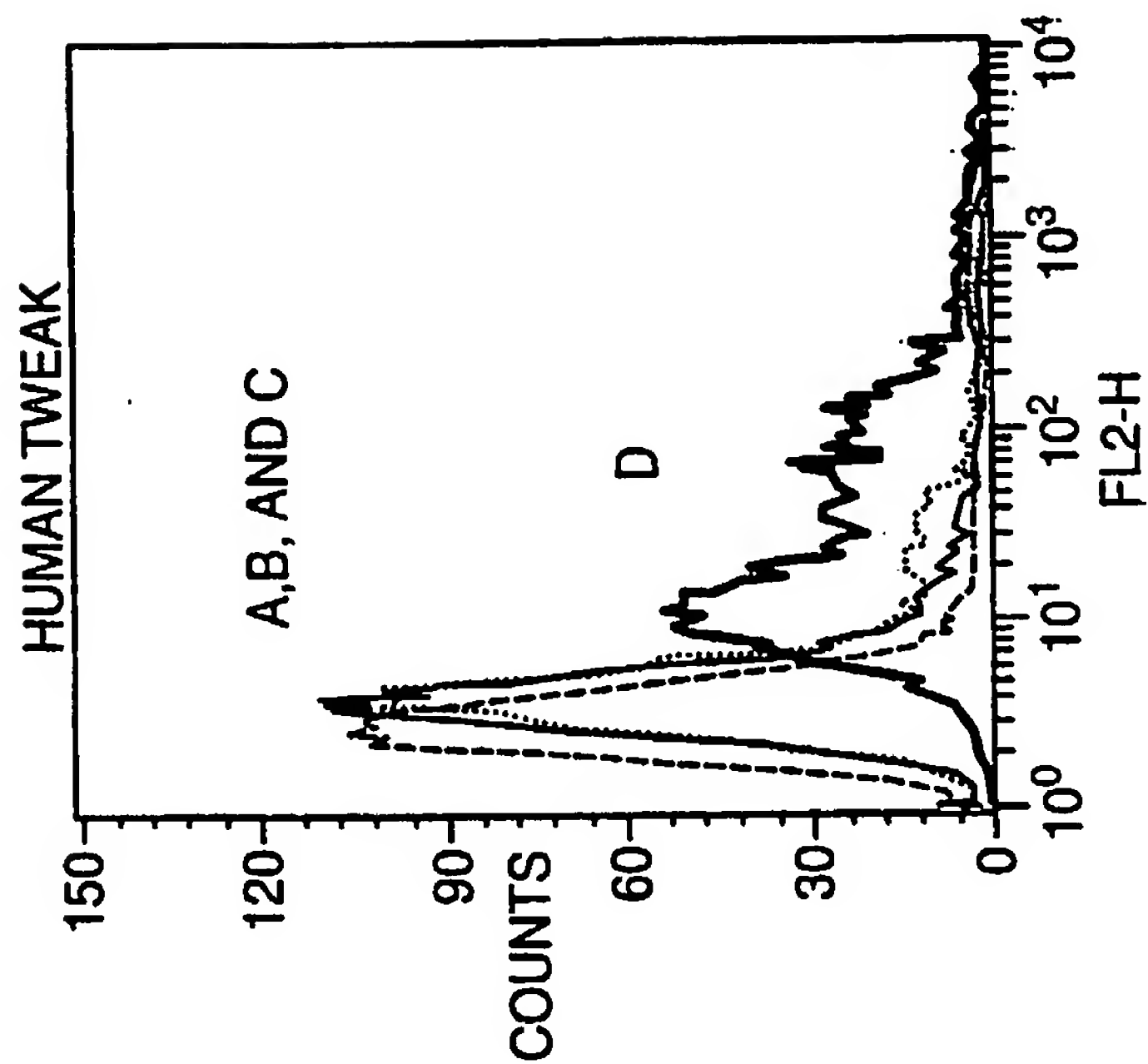


FIG. 2d

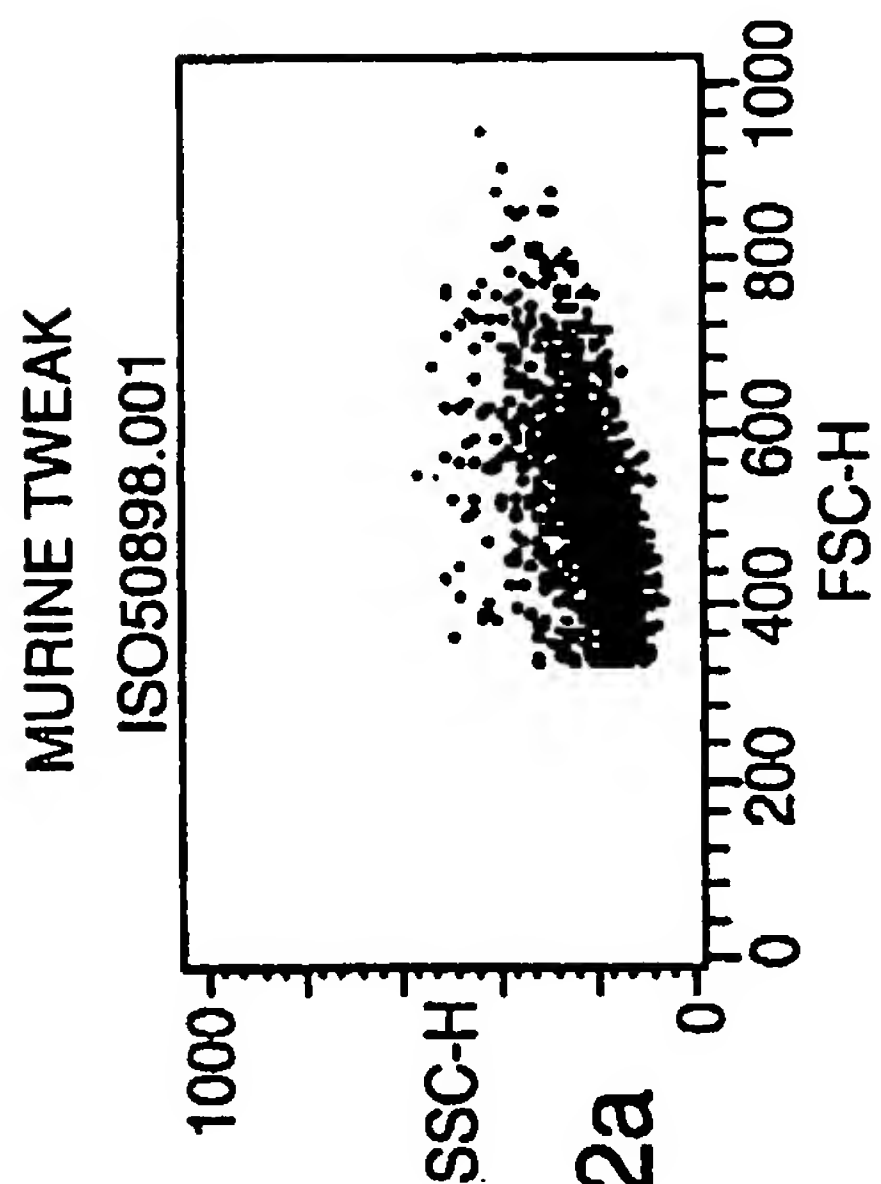


FIG. 2a

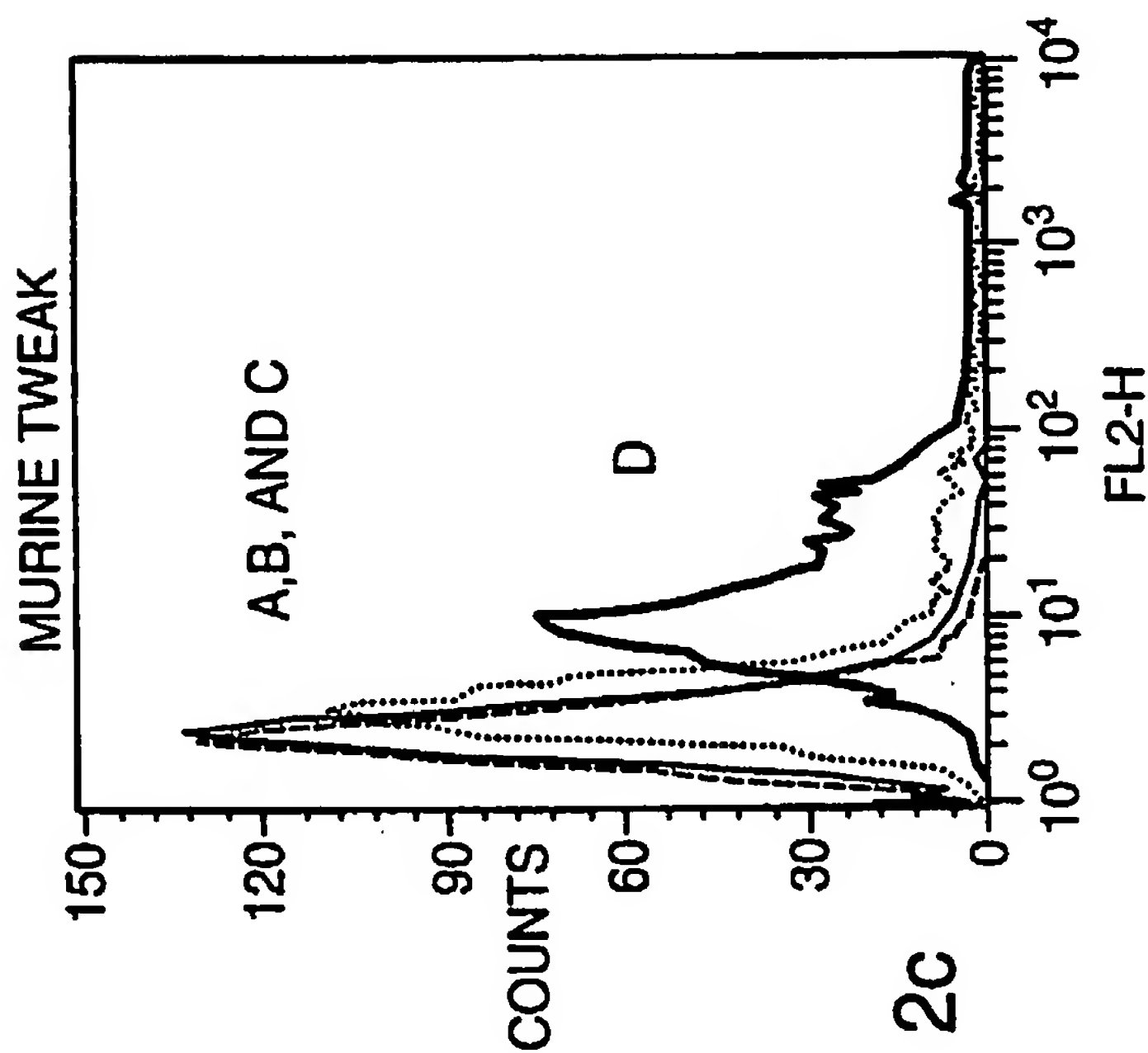


FIG. 2c

3/5

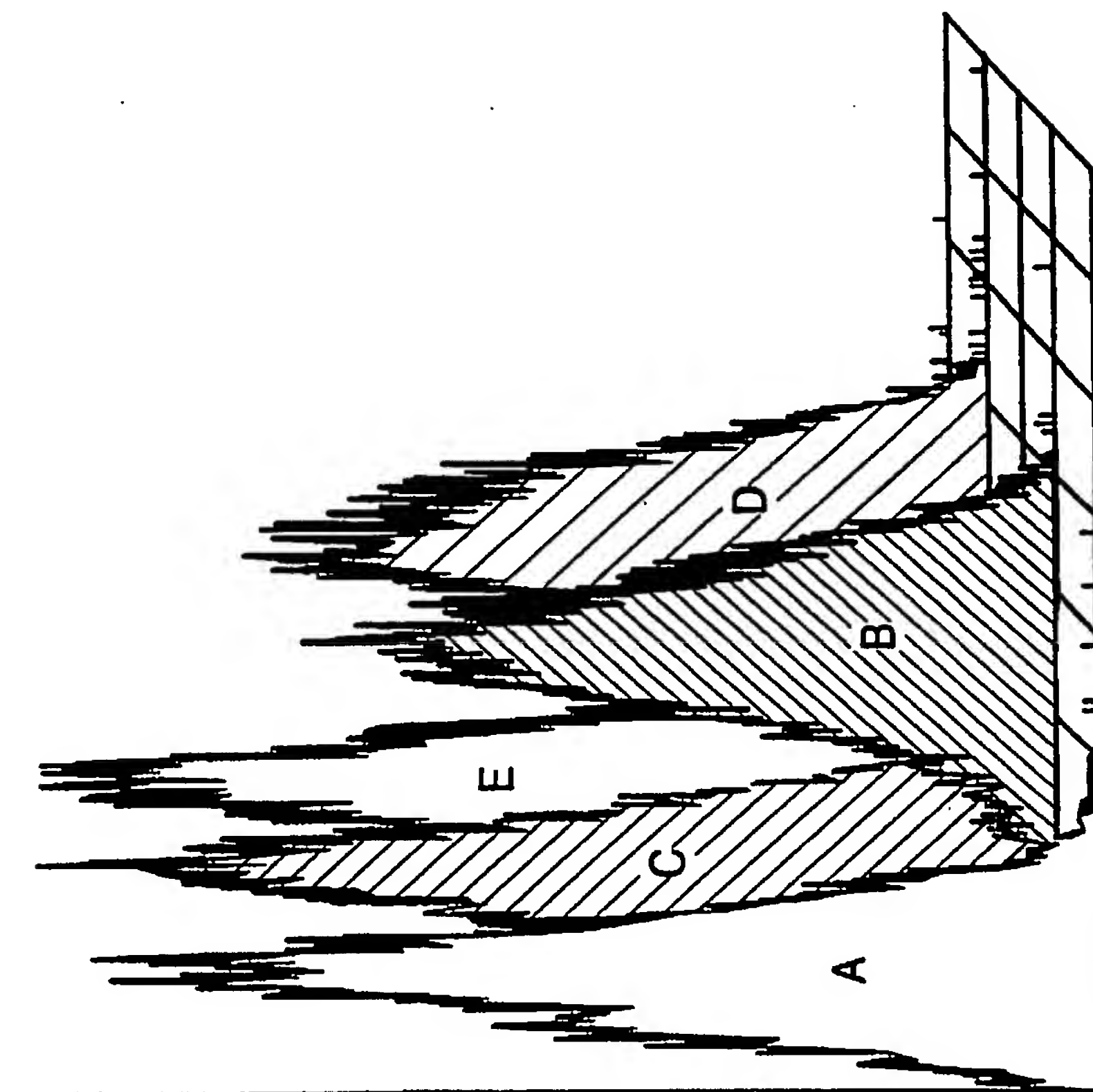


FIG. 3b

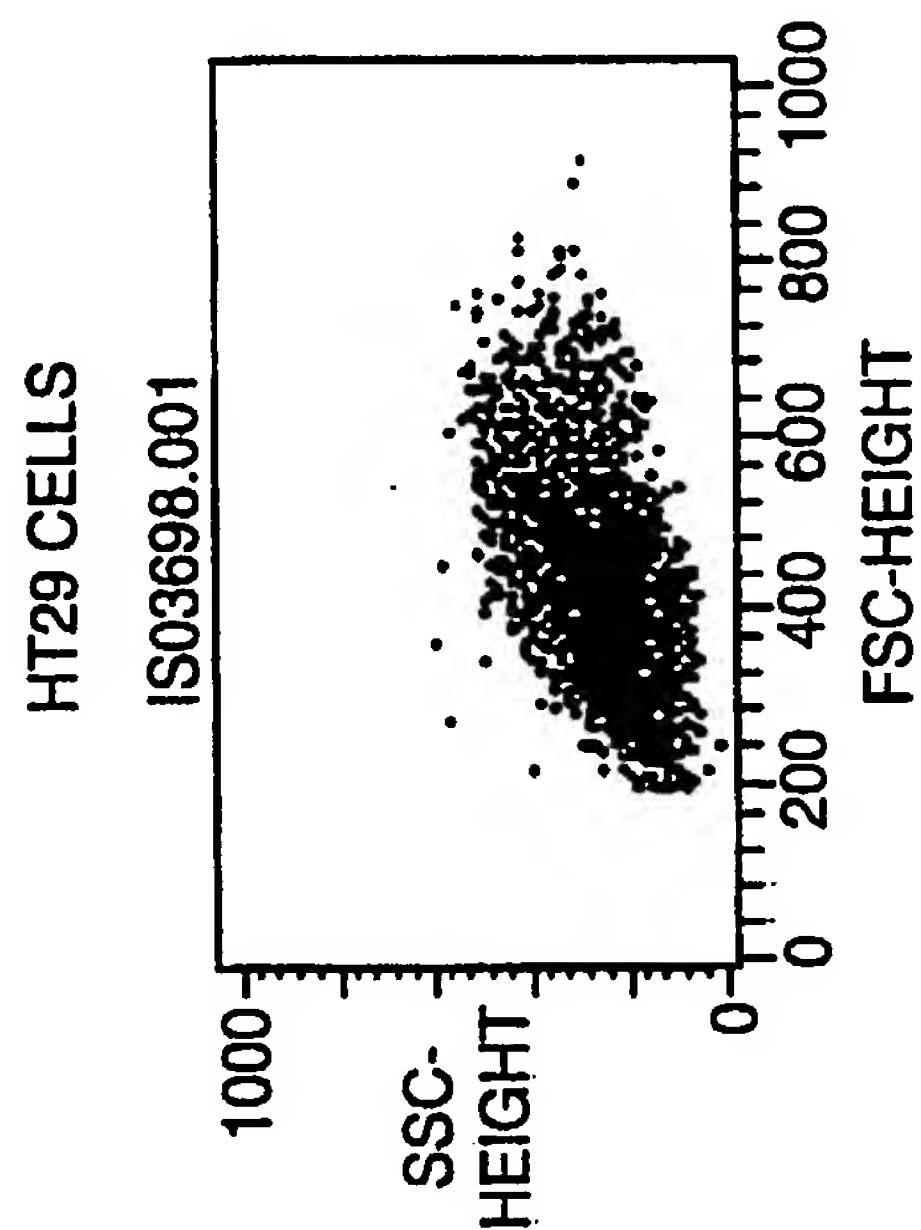


FIG. 3a



FIG. 4a

FIG. 4b

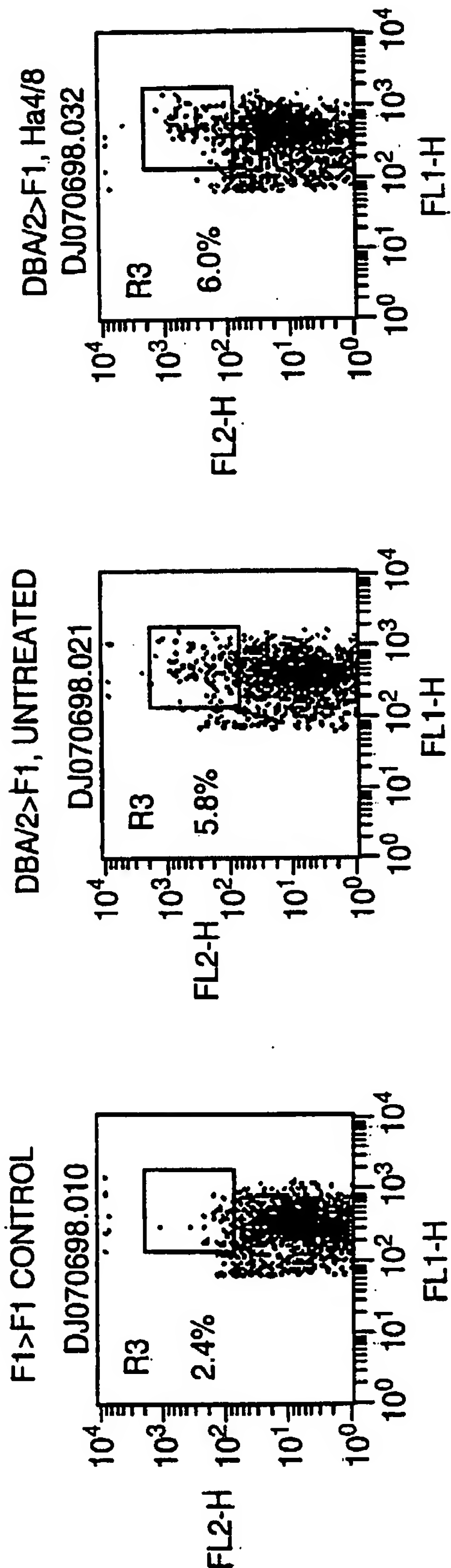


FIG. 4c

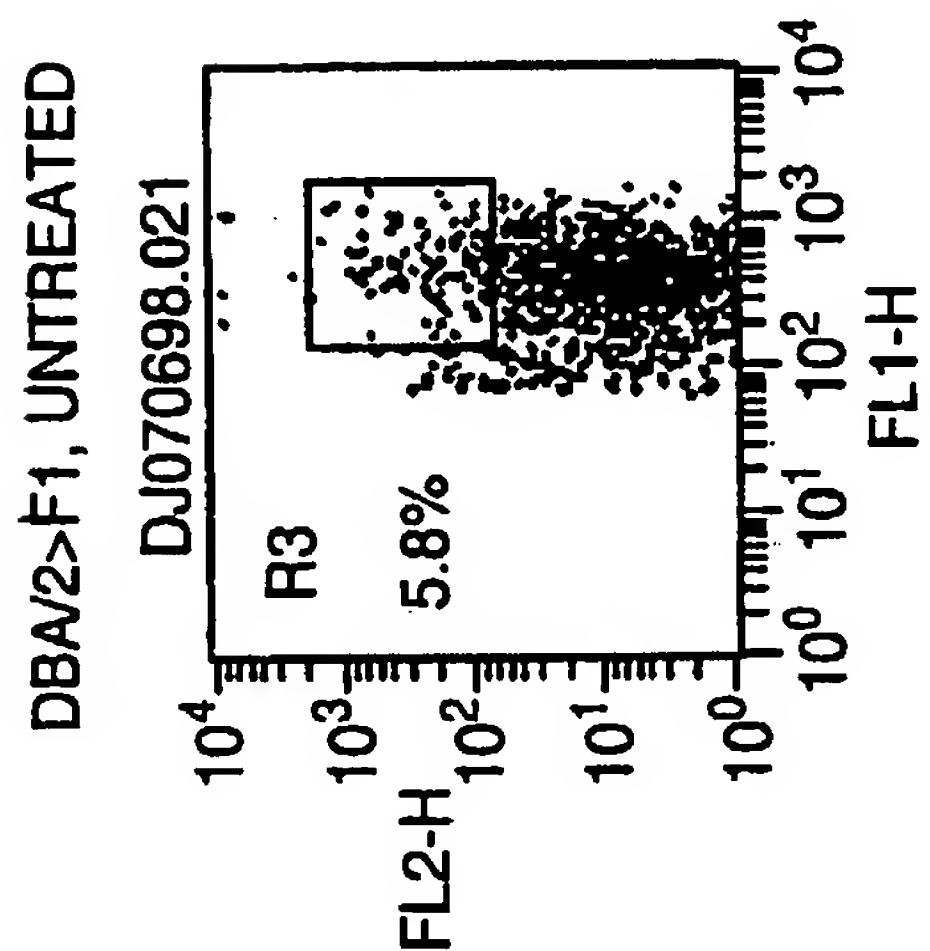


FIG. 4d

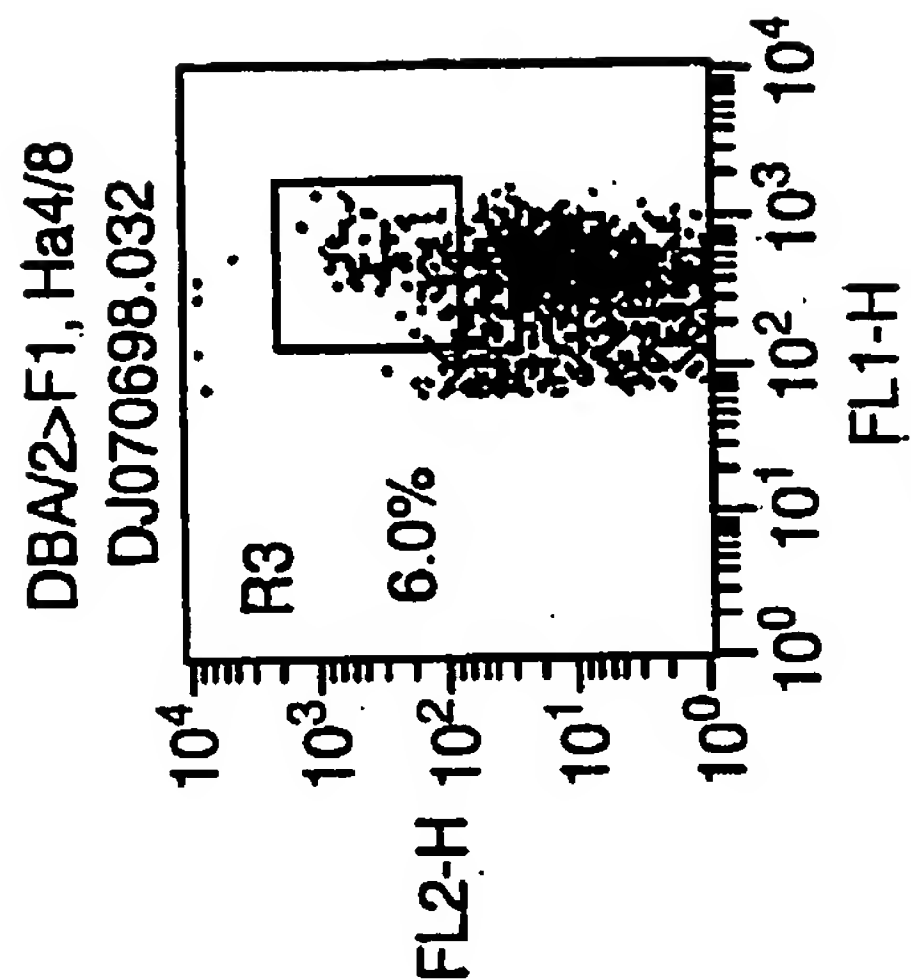


FIG. 4e

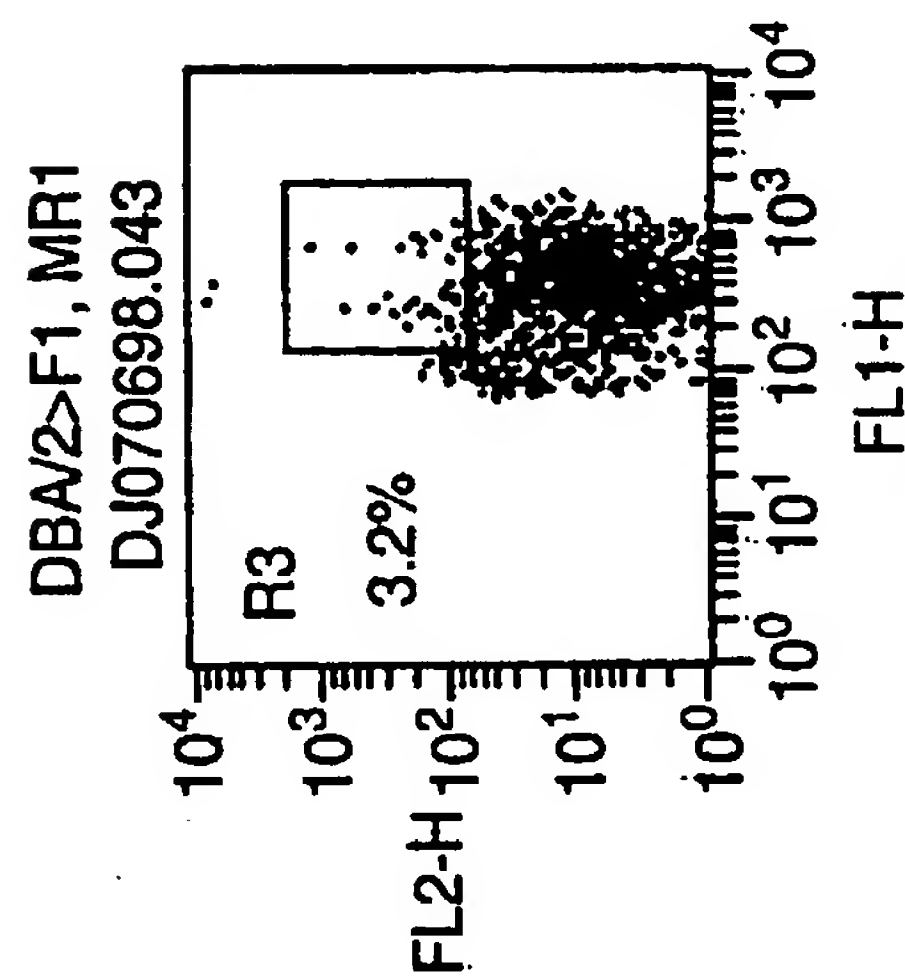


FIG. 4f

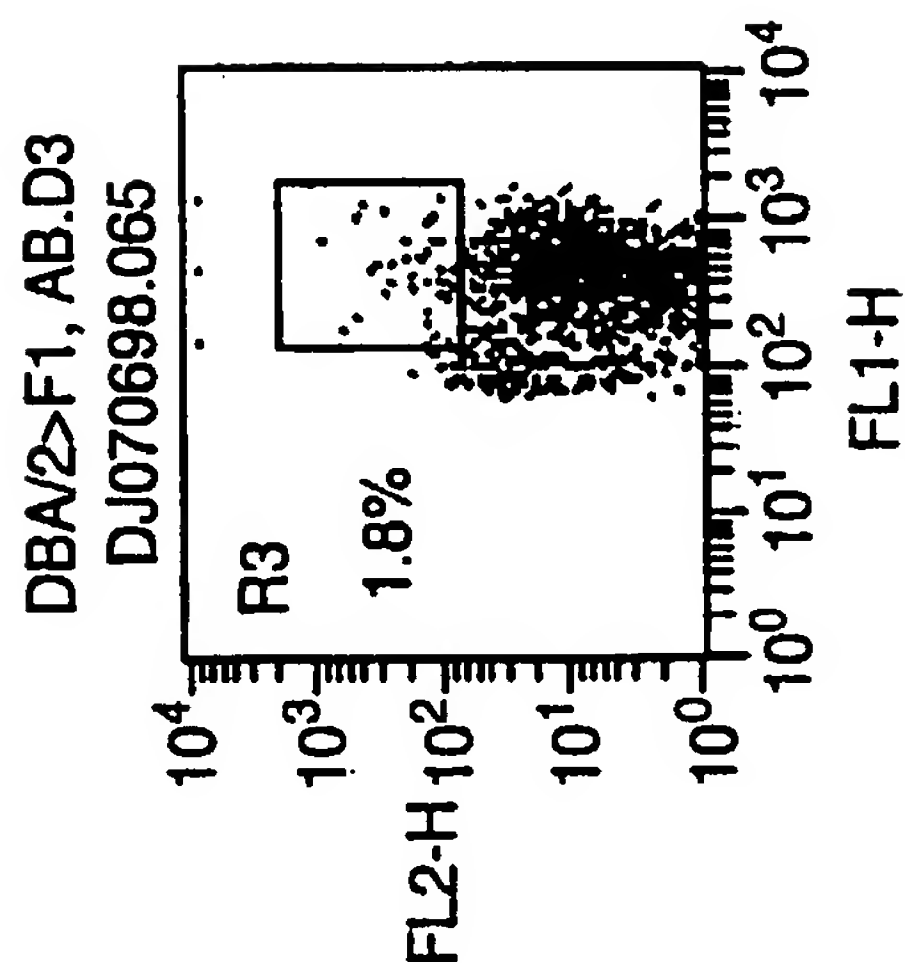


FIG. 4g

# INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 00/01044

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/28 A61K38/17 A61K39/395 A61P37/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 05783 A (BIOGEN) 12 February 1998 (1998-02-12) claims 1-35	1-22
X	WO 98 35061 A (ABBOTT LABORATORIES) 13 August 1998 (1998-08-13) claim 24	16, 21, 22
P, X	WO 99 19490 A (GENENTECH) 22 April 1999 (1999-04-22)  claims 1-29	1-5, 10-14, 16, 21, 22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

29 May 2000

Date of mailing of the international search report

06/06/2000

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 00/01044

### Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/US 00/01044

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9805783 A	12-02-1998	AU 3829497 A BR 9711046 A CN 1232503 A CZ 9900403 A EP 0956351 A NO 990550 A PL 331583 A	25-02-1998 11-01-2000 20-10-1999 11-08-1999 17-11-1999 06-04-1999 19-07-1999
WO 9835061 A	13-08-1998	EP 0977887 A	09-02-2000
WO 9919490 A	22-04-1999	AU 1077099 A	03-05-1999